0104/02

JC13 Rec'd PCT/PTO 09 NOV 2001

FORM PTO-1390 (REV. 10-2000)	U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE	ATTORNEY'S DOCKET NUMBER 20455P
TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371		U.S. APPLICATION NO. (If known, see 37 CFR 1.5) 10/030378
INTERNATIONAL APPLICATION NO. PCT/US00/12638	INTERNATIONAL FILING DATE 10 MAY 2000	PRIORITY DATE CLAIMED 14 MAY 1999

TITLE OF INVENTION
DETECTION OF VIRAL STABILITY

APPLICANT(S) FOR DO/EO/US
JEFFREY T. BLUE

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. [X] This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. [ ] This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. [ ] This is an express request to begin national examination procedures [35 U.S.C. 371(f)] at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(l).
4. [X] A proper Demand for International Preliminary Examination was made and the US was elected by the expiration of the 19th month from the earliest claimed priority date (PCT Article 31).
5. [X] A copy of the International Application as filed [35 U.S.C. 371(c)(2)].
   a. [X] is attached hereto (required only if not communicated by the International Bureau).
   b. [ ] has been communicated by the International Bureau.
   c. [ ] is not required, as the application was filed in the United States Receiving Office (RO/US).
6. [ ] An English language translation of the International Application as filed [35 U.S.C. 371(c)(2)].
7. [ ] Amendments to the claims of the International Application under PCT Article 19 [35 U.S.C. 371(c)(3)].
   a. [ ] are attached hereto (required only if not communicated by the International Bureau).
   b. [ ] have been communicated by the International Bureau.
   c. [ ] have not been made; however, the time limit for making such amendments has NOT expired.
   d. [ ] have not been made and will not be made.
8. [ ] An English language translation of the amendments to the claims under PCT Article 19 [35 U.S.C. 371(c)(3)].
9. [X] An oath or declaration of the inventor(s) [35 U.S.C. 371(c)(4)].
10. [ ] An English language translation of the annexes to the International Preliminary Examination Report under PCT Article 36 [35 U.S.C. 371(c)(5)].

Items 11 to 16 below concern other document(s) or information included:

11. [X] An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
12. [X] An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
13. [X] A **FIRST** preliminary amendment.
    [ ] A **SECOND** or **SUBSEQUENT** preliminary amendment.
14. [ ] A substitute specification.
15. [ ] A change of power of attorney and/or address letter.
16. [ ] Other items or information:

EXPRESS MAIL CERTIFICATE
DATE OF DEPOSIT 11-9-2001
EXPRESS MAIL NO. EM230077767 US
I HEREBY CERTIFY THAT THIS CORRESPONDENCE IS BEING DEPOSITED WITH THE UNITED STATES POSTAL SERVICE AS EXPRESS MAIL "POST OFFICE TO ADDRESSEE" BEFORE 5 P.M. ON THE ABOVE DATE IN AN ENVELOPE ADDRESSED TO ASSISTANT COMMISSIONER FOR PATENTS, WASHINGTON, D.C. 20231.
MAILED BY A. D. Crowley
DATE 11-9-01

Computer generated form "TransLetter (DO-EO-US)" (PCT Folder), Merck & Co., Inc., 10/01/01

U.S. APPLICATION NO. (If known, see 37 CFR 1 5)	INTERNATIONAL APPLICATION NO	ATTORNEY'S DOCKET NUMBER
10/030378	PCT/US00/12638	20455P

17. ☒ The following fees are submitted:	CALCULATIONS	PTO USE ONLY
BASIC NATIONAL FEE [37 CFR 1.492(a)(1)-(5)]:		
Neither international preliminary examination fee (37 CFR 1.482) nor international search fee [37 CFR 1.445(a)(2)] paid to USPTO and International Search Report not prepared by the EPO or JPO **$1,040.00**		
International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO..... **$890.00**		
International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee [37 CFR 1.445(a)(2)] paid to USPTO..... **$740.00**		
International preliminary examination fee paid to USPTO (37 CFR 1.482) but all claims did not satisfy provisions of PCT Article 33(1)-(4)..... **$710.00**		
International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(1)-(4)..... **$100.00**		
ENTER APPROPRIATE BASIC FEE AMOUNT =	$100.00	
Surcharge of **$130.00** for furnishing the oath or declaration later than ☐ 20 ☐ 30 months from the earliest claimed priority date [37 CFR 1.492(e)].	$0.00	

Claims	Number Filed	Number Extra	Rate		
Total Claims	17 - 20 =	0	X **$18.00**	$0.00	
Independent Claims	2 - 3 =	0	X **$84.00**	$0.00	
Multiple dependent claim(s) (if applicable)			+ **$280.00**	$0.00	
TOTAL OF ABOVE CALCULATIONS =				$100.00	
☐ Applicant claims small entity status. See 37 CFR 1.27. The fees indicated above are reduced by 1/2.					
SUBTOTAL =				$100.00	
Processing fee of **$130.00** for furnishing the English translation later than ☐ 20 ☐ 30 months from the earliest claimed priority date [37 CFR 1.492(f)]. +				$0.00	
TOTAL NATIONAL FEE =				$100.00	
Fee for recording the enclosed assignment [37 CFR 1.21(h)]. The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). **$40.00** per property. +					
TOTAL FEES ENCLOSED =				$100.00	
				Amount to be refunded	
				charged	

a. ☐ A check in the amount of $ _____ to cover the above fees is enclosed.

b. ☒ Please charge my Deposit Account No. 13-2755 in the amount of $100.00 to cover the above fees. A duplicate copy of this sheet is enclosed.

c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to the Deposit Account No. 13-2755. A duplicate copy of this sheet is enclosed.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive [37 CFR 1.137(a) or (b)] must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

MERCK & CO., INC.
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DATE: November 9, 2001

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SHELDON O. HEBER
NAME

38,179
REGISTRATION NUMBER

10/030303.8

PATENT

JC10 Rec'd PCT/PTO 09 NOV 2001

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant	Blue, J
Serial No.:	TBA - Case No.: 20455P
Filed:	November 9, 2001
For:	DETECTION OF VIRAL STABILITY

Art Unit:

Examiner:

PRELIMINARY AMENDMENT

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

Please amend the specification of the present application on page 1, line 3, under the title of the invention, by adding:

--PRIORITY

The present application claims priority to provisional application U.S. Serial Number 60/134,163 filed May 14, 1999.--.

Please charge deposit account 13-2755 if any fees are due in connection with this amendment.

Respectfully submitted,

EXPRESS MAIL CERTIFICATE
DATE OF DEPOSIT 11-9-2001
EXPRESS MAIL NO. EM230307767US
I HEREBY CERTIFY THAT THIS CORRESPONDENCE IS BEING DEPOSITED WITH THE UNITED STATES POSTAL SERVICE AS EXPRESS MAIL "POST OFFICE TO ADDRESSEE" BEFORE 5 P.M. ON THE ABOVE DATE IN AN ENVELOPE ADDRESSED TO ASSISTANT COMMISSIONER FOR PATENTS, WASHINGTON, D.C. 20231.
MAILED BY [signature]
DATE 11/9/01

By [signature]

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TITLE OF THE INVENTION
DETECTION OF VIRAL STABILITY

FIELD OF THE INVENTION

The present invention pertains to assays for measuring viral potency and stability and for evaluating the stability of a virus in different formulations.

BACKGROUND OF THE INVENTION

The references cited herein are not admitted to be prior art to the claimed invention.

In developing live virus vaccines it is important to determine viral potency and stability to assure adequate immunization. The plaque assay is a common method for determining viral potency and stability. The plaque assay is often used to examine the effect of different storage conditions on viral stability.

The plaque assay involves inoculating viruses onto a cell sheet at dilutions determined by the expected (estimated) potency. The plates containing the infected cells are incubated for a specified length of time and then stained. Viral infection results in plaque formation, or areas of dead or detached cells. Counting the number of plaques gives the corresponding plaque-forming units (PFU), a measure of potency. Viral stability is determined by examining the change in PFU over time.

SUMMARY OF THE INVENTION

Viral induction of caspase 3 activity was found to provide a reliable measure of viral activity. Assaying viral induction of caspase 3 activity can be used, for example, in methods for measuring viral potency and stability, and for evaluating the stability of a virus in different formulations.

Thus, a first aspect of the present invention describes a method of assaying the potency and stability of a virus by measuring viral induction of caspase 3 activity. The method involves infecting a plurality of cells susceptible to caspase 3 induction with the virus and measuring caspase 3 activity as an indication of viral potency and stability. A plurality of cells is a population of the same strain of cells.

Another aspect of the present invention describes a method of identifying a stabilizing formulation for a virus. The method involves infecting a strain of cells susceptible to caspase 3 induction with a virus in different test formulations. The

strain of cells is provided as different pluralities of cells where each plurality of cells is infected with the virus. Induction of caspase 3 activity produced from virus stored in the different formulations is measured to identify the formulations that stabilize the virus.

Other features and advantages of the present invention are apparent based on the descriptions provided herein. The examples provided herein illustrate different components and methodology useful in practicing the present invention. The examples do not limit the claimed invention. Based on the present disclosure the skilled artisan can identify and employ other components and methodology for practicing the present invention.

BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1a and 1b illustrate induction times for caspase 3 activity using lyophilized virus and liquid bulk virus (*i.e.*, a concentrated lot of clarified virus). The RFU (relative fluorescent unit) value was adjusted by subtracting background signal (adjusted RFU). Figure 1a illustrates an induction time using lyophilized mumps virus ("MuV"), lyophilized rubella virus ("RbV"), and lyophilized measles virus ("MeV"). Figure 1b illustrates an induction time using bulk measles virus.

Figures 2a and 2b illustrate a linear range for induction of caspase 3 activity. Figure 2a provides results using measles virus. Figure 2b provides results using mumps virus.

Figure 3 illustrates measles virus induction variation in various samples. "Liquid" indicates a non-lyophilized frozen aliquot. "D0" indicates a lyophilized non-incubated sample stored at -70°C. The one-week sample was initially lyophilized and stored at 37°C for one week. "A", "C", "D" and "F" refer to different formulations of the vaccine.

Figure 4 illustrates a comparison of the plaque assay and the caspase 3 assay. Activity was measured using measles virus stored in different environments. "Liq" indicates a non-lyophilized frozen aliquot. "D0" indicates a lyophilized, non-incubated sample stored at -70°C. "D7" indicates a lyophilized sample incubated at 37°C for one week. "A", "B", "C" and "D" refer to different formulations.

Figure 5 illustrates a comparison of the plaque assay and the caspase 3 assay. Viral activity was measured using mumps virus stored in different environments. "Liq" indicates a non-lyophilized frozen aliquot. "D0" indicates a lyophilized non-

incubated sample stored at -70°C. "D7" indicates a lyophilized sample incubated at 37°C for one week. "A", "B", "C" and "D" refer to different vaccine formulations being tested.

Figure 6 illustrates a comparison of the plaque assay and the caspase 3 assay. Viral activity was measured using rubella virus stored in different environments. "Liq" indicates non-lyophilized frozen aliquot. "D0" indicates a lyophilized, non-incubated sample stored at –70°C. "D7" indicates lyophilized sample incubated at 37°C for one week. "H", "I", "J", "K" and "L" refer to different formulations of the vaccine.

DETAILED DESCRIPTION OF THE INVENTION

Measuring viral induction of caspase 3 activity was found to provide a reliable measure of viral potency and stability. Useful properties of measuring viral induction of caspase 3 activity include: induction of caspase 3 activity is achieved by different viruses including measles, mumps and rubella viruses; induction of caspase 3 activity is reproducible; induction of caspase 3 activity is sensitive to viral dilutions; induction of caspase 3 activity is sensitive to sample differences; and induction of caspase 3 activity correlates with the plaque assay. Thus, the caspase 3 assay can be applied to different formulations and a variety of viruses. Additionally, caspase 3 activity is not substantially effected by freezing and thawing, can be specifically detected, and is quenchable. These advantages allow the assay to be easily implemented for routine laboratory handling.

Another advantage of the caspase 3 assay in time. When measuring viral stability, the caspase 3 assay can be performed quicker than the plaque assay. The standard plaque assay takes approximately seven to ten days, but the caspase 3 assay can be performed in about half that time.

Caspase 3

Caspase 3 (also known as CPP32 or apopain) is a member of the caspase family of proteases. Caspases are induced by apoptosis, which is an active process of cellular suicide. (*Thompson, Science 267*:1456-1462, 1995; Nicholson, *Nature Biotechnology 14*:297-301, 1996; Lincz, *Immunology and Cell Biology 76*:1-19, 1998.) Apoptosis has been indicated to be induced by different stimuli including infection by different viruses. (Shen, *et al., Current Opinion in Genetic and*

Development 5:105-111, 1995; Sadzot-Delvaux, *et al., Journal of General Virology* 76:2875-2879, 1995; Esolen, *et al., Journal of Virology* 69:3955-3958, 1995; and Ito, *et al., FEMS Immunology and Medical Microbiology* 15:115-122, 1996.) However, certain viral proteins have been indicated to block apoptosis, such as Baculovirus protein p35 which inhibits caspase 3. (Lincz, *Immunology and Cell Biology* 76:1-19, 1998.)

Caspase 3 is a cysteine protease synthesized from a pro-enzyme by cleavage at an Asp residue to form an active protease. The active caspase 3 protease also cleaves at Asp residues. The specific recognition sequence for caspase 3 is the peptide Asp-Glu-Val-Asp. (Fernandes-Alnemri, *et al., Journal of Biological Chemistry* 269:30761-30764, 1994; Nicholson, *et al., Nature* 376:37-43, 1995; Srinivasula, *et al, Journal of Biological Chemistry* 271:27099-27106, 1996; Schlegel, *et al., Journal of Biological Chemistry* 271:1841-1844, 1996; and Casciola-Rosen, *et al., J. Exp. Med.* 183:1957-1964, 1996; each of these references is hereby incorporated by reference herein.)

Selection of Appropriate Viruses and Cells

Suitable virus and cells for use in the present invention can be selected based upon the susceptibility of a particular cell to caspase 3 induction when infected with a particular virus. The ability of a virus to infect a particular cell can readily be determined, as can the ability of the virus to induce caspase 3 activity. Examples of procedures that can be used to measure the suitability of a cell and a virus are described in the Example section provided below using measles, mumps and rubella virus. Other cells (*e.g.*, MRC-5) and viruses (*e.g.*, varicella-zoster) are expected to be useful in the present invention.

Measuring Caspase 3 Activity

"Caspase 3 activity" refers to enzymatic activity able to cleave the caspase 3 substrate Asp-Glu-Val-Asp ("DEVD"). Such activity is known to be produced by caspase 3 and at least one related enzyme, Mch3α. (*Fernandes-Alnemri, et al., Cancer Research* 55:6045-6052, 1995, which is hereby incorporated by reference herein.)

Based on the present disclosure, caspase 3 activity induced by viral infection can be measured using techniques well known in the art. Preferably, caspase 3

activity is measured using colorimetric or fluorimetric labeled substrates. More preferably, the employed substrate is DEVD linked to a colorimetric or fluorimetric moiety. Examples of such a moiety include the colorimetric moiety *p*-nitroanilide (λ_{max} = 505 nm) and the fluorimetric moiety 7-amino-4-trifluoromethyl coumarin ("AFC", λ_{max} = 400 nm). (Zhang, *et al.*, in *Apoptosis Detection and Assay Methods,* pages 7-14, Eds. Zhu and Chun, *BioTechniques Books*, 1998, both of which are hereby incorporated by reference herein). Colorimetric and fluorimetric labeled substrates can be employed using procedures such as those described by Zhang, *et al.*, in *Apoptosis Detection and Assay Methods,* pages 7-14, Eds. Zhu and Chun, *BioTechniques Books*, 1998; and ApoAlert™ CPP32/Caspase-3 Assay Kits User Manual (PT3083-1), CLONTECH Laboratories, Inc. 1998; both of which are hereby incorporated by reference herein.

The caspase 3 assay is preferably employed on viral vaccine samples either in liquid or lyophilized form. For example, lyophilized viral samples can be reconstituted, diluted, and plated onto a cell sheet; the samples are incubated, the cells are lysed, and the cellular lysate collected and frozen at –70°C; after thawing on wet ice (about 5°C) removal of cellular debris by centrifugation occurs, the supernatant is removed to a new tube containing reaction buffer, and the DEVD-AFC substrate is incubated with the cellular supernatant.

EXAMPLES

Examples are provided below to further illustrate different features and advantages of the present invention. The examples also illustrate useful methodology for practicing the invention. These examples do not limit the claimed invention.

Methods and Materials

Methods and materials generally used in growing cells, inducing caspase 3 activity, and measuring caspase 3 activity to obtain the results provided below were as follows:

Growth of Vero Cells and RK-13 Cells

Vero cells were grown to confluency in 162 cm^2 flasks in M199 media (Gibco/BRL cat# 11150-059) containing 10% fetal bovine serum (FBS) and 0.5% neomycin at 35°C with 5% CO_2. The flasks were then decanted and 3 ml of 1x

trypsin-ethylene diamine tetra acetic acid (EDTA) (Gibco/BRL cat# 25200-056) was added to the flasks to neutralize the media. The trypsin was decanted and another 2 mls of trypsin was added and incubated for 10 to 15 minutes at 35°C with 5% CO_2. Cells were then washed to the bottom of the flask with 20 ml of M199 containing 10% FBS and 0.5% neomycin.

Cells were plated into 24 well plates at 5.4 X 10^4 cells/ml. Each well received 2 ml of the diluted cells (diluted into M199 containing 10% FBS and 0.5% neomycin). The plates were incubated at 35°C for 2 days and then used for caspase 3 induction. The growth of RK-13 cells is the same as Vero cells with the exception of the media. RK-13 cells were grown in MEM media (Gibco/BRL) containing 10% FBS and 0.5% neomycin. The cells were plated at a density of 6.0 x 10^4 cells /ml, 2 ml per well.

Induction of Caspase 3 Activity

Generally, Vero plates were used for measles virus and mumps virus infections, while RK-13 cells were used for the rubella virus infections. Lyophilized formulations of a combination of measles, mumps, and rubella vaccine were used. Lyophilized formulations were reconstituted with 0.7 ml dH_2O. Frozen liquid samples were thawed at room temperature prior to use. Following reconstitution or liquid thawing, the samples were diluted 1:2, 1:5, 1:10 or 1:20 in their respective diluents. The diluents contained antibodies against the viruses not being assayed for among mumps, measles, and rubella viruses (*e.g.,* when assaying for measles virus antibodies for mumps and measles virus were used).

Cells ($2.0x10^5$) were infected with 50 µl of the diluted samples. Each sample was used to infect 6 wells on a 24 well plate. Plates were then incubated at 35°C with 5% CO_2 for 1 hr for plates to be assayed for mumps and rubella viruses and for 2 hrs for plates to be assayed for measles virus. During this attachment phase, the plates were rocked every 15 minutes to ensure proper coverage of the cell sheet with media. Following the attachment phase, the cells were overlayed with 1 ml of M199 media containing 10% FBS and 0.5% neomycin for the Vero cells and 1 ml of MEM containing 10% FBS and 0.5% neomycin for the RK-13 cells. The plates were then incubated at 35°C with 5% CO_2 for 93 to 96 hrs.

Caspase 3 Assay

Following the induction period, media was aspirated from the wells. Thirty microliters of chilled cell lysis buffer (Clontech: 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X –100) was added per well. The samples were incubated on ice for 10 minutes and then two wells were pooled together into one microfuge tube. Samples were either frozen on dry ice and stored at –70°C until time of analysis or were assayed immediately.

The samples were spun in at 12,000 rpm for 5 minutes at 4°C. Supernatant (50 µl) was removed to a new microfuge tube and 50 µl of 2x Reaction Buffer (Clontech: 100 mM HEPES, pH 7.4, 150 mM NaCl, 0.2% CHAPS) containing 7 mM dithiothreitol (DTT) was added. Five microliters of the 1 mM DEVD-AFC substrate was added to the reaction and vortexed briefly. The samples were then incubated for 1 hr at 37°C in a circulating water bath. Following the incubation, 20 µl of 10% H_3PO_4 was used to quench the reaction. Quenched reactions (100 µl) were added to a 96 well plate and read in a TECAN fluorescent plate reader (excitation: 390 nm, emission: 480 nm, gain: 60).

Results

Detection of Caspase 3 Activity Induced by Measles, Mumps and Rubella Viruses

The ability of measles, mumps, and rubella viruses to induce caspase 3 activity in cells was confirmed using Vero and RK-13 cells. Caspase 3 activity was measured as described above in Methods and Materials. Vero cells were induced with measles virus, mumps virus, and rubella virus, while RK-13 cells were induced with rubella virus. The results are shown in Table 1

Table 1

	Induced	Non-Induced
Measles Virus	12861	1210
Mumps Virus	20085	1286
Rubella Virus	4060	554

Caspase 3 activity expressed as RFU in induced (*i.e.* infected) cells and non-induced controls.

To ensure that the observed signal was due to the virus, one set of Vero cells were infected with measles virus and another set of Vero cells were infected with a placebo. The placebo produced an adjusted RFU of 50-500, while measles virus produced an adjusted RFU of about 7,750. These results demonstrate that significant changes in caspase 3 activity are due to viral infection rather than a formulation component.

Effects of Freeze-Thawing on Caspase 3 Activity

To allow an assay to be run at various times after infected, samples may be frozen and later thawed at a convenient time. To determine if freezing and thawing affects caspase 3 activity, samples were frozen on dry ice and stored at –70°C following cell lysis. The samples were then thawed and assayed with a set of samples that were not frozen. Non-frozen cells provided an adjusted RFU of 2,167 while frozen cells produced an adjusted RFU of 2,175.5. Thus, freeze-thawing does not appear to effect caspase 3 activity. Accordingly, many samples can be run and then stored for various lengths of time allowing the actual assay to be run at a later time.

Determination of the Optimal Time Required for Viral Bulk Incubation

The time required to induce caspase 3 activity with various samples can differ depending on the virus and multiplicity of infection (MOI). To determine preferred times for measles, mumps and rubella virus infection, Vero cells or RK-13 cells were infected with (1) measles virus bulk and (2) different lyophilized measles virus, mumps virus, and rubella virus. Vero cells were infected with mumps and measles, while RK-13 cells were infected with rubella virus. A long-term study was initiated with time points collected at 24, 46, 72, and 96 hours for bulk samples and 26, 47, 75, 94, 95, 97 and 100 hours for lyophilized samples.

The results of different points are presented in Figures 1a and 1b. All three viruses in lyophilized formulations show a peak signal around 95 hours (Figure 1a). The optimal signal observed for the infection with measles virus bulk was around 46 hours (Figure 1b). This difference in overall time is expected since a bulk sample contains a much higher titer and infects more cells upon inoculation, thereby decreasing the length of time necessary to ensure an optimal signal.

Linearity of the Caspase 3 Enzyme Response from Cells induced with Measles Virus and Mumps Virus

When investigating and comparing enzyme activity, it is important to collect data found within the linear range of the assay. Following induction with measles and mumps viruses, the samples were assayed and data collected over a one-hour period for the measles virus and an hour and a half for mumps virus. The data presented in Figure 2a (measles virus) shows the assay is linear for at least one hour, and the data presented in Figure 2b (mumps virus) show the assay is linear for at least 75 minutes. Thus, using a one-hour incubation period for caspase 3 enzyme activity will ensure the data collected will be meaningful.

Determination of Non-Specific Substrate Cleavage

A concern with using a non-purified cell lysate is the effect of non-specific substrate cleavage derived from other proteases found within the lysate. Using a specific inhibitor for caspase 3, DEVD-CHO, the caspase 3 assay was run and analyzed. If non-specific cleavage were occurring in the assay, the addition of the inhibitor would not completely stop the substrate from being cleaved. The results shown in Table 2 indicate that adding 1μM DEVD-CHO inhibitor eliminates the RFU signal.

TABLE 2

Virus	- Inhibitor (RFU)	+ Inhibitor (RFU)
Measles Virus	2538	6
Rubella Virus	3205	10
Mumps Virus	3074	0

RFU was adjusted to take background into account.

Samples without the caspase 3 inhibitor show a ~3,000 fold increase over the samples containing the inhibitor. Thus, non-specific cleavage is not occurring in these cellular lysates and the signal is derived from caspase 3 activity.

The Effects of Quenching Caspase 3 Assay using 10% H_3PO_4

To ensure consistent data when using enzymes, it is useful to be able to quench the reaction. Quenching can be used to ensure that each sample is incubated for the same amount of time. It is useful to examine the quenching conditions to ensure that the quench is not having an effect on the RFU signal that is being generated. In some instances, quenching a fluorescent peptide will provide interference on the overall signal making data interpretation difficult.

Following a one hour incubation period, samples were quenched with 20 µl of 10% H_3PO_4 and analyzed on the TECAN Plate reader. Data were compared with that obtained from samples that did not undergo quenching. The results are shown in Table 3.

TABLE 3

	Not Quenched (RFU)	Quenched (RFU)	Non-Induced Not Quenched (RFU)	Non-Induced Quenched (RFU)
Measles Virus	9331	5069	1046	508

The results indicate the ratio of the non-induced samples to the induced samples is the same. Thus, quenching the reactions does not effect the relative signal intensity.

Reproducibility of the Caspase 3 Assay

Using samples induced by measles virus, mumps virus, or rubella virus, the reproducibility of measuring caspase 3 activity was examined by using three vials of the same sample. The data presented in Table 4 show that the assay is reproducible and very consistent.

TABLE 4

	Sample 1 (RFU)	Sample 2 (RFU)	Sample 3 (RFU)
Measles Virus	4332	4372	3694
Mumps Virus	11377	12621	11454
Rubella Virus	3248	3166	3199

RFU was adjusted to take into account the background.

Effects of Dilutions on Caspase 3 Signal

To produce a quantitative assay it is important to detect differences in signal when the sample is diluted to different multiplicity of infections. To address this, measles virus and rubella virus lyophilized samples were diluted 1:2, 1:5 and 1:10 in their respective diluents, and mumps virus was diluted 1:20, 1:40, and 1:80, and the effect on caspase 3 signal was measured. The results are shown in Tables 5 and 6.

TABLE 5

	1:2 Dilution (RFU)	1:5 Dilution (RFU)	1:10 Dilution (RFU)
Measles Virus	8221	3112	1441
Rubella Virus	3184	1721	799

RFU was adjusted to take into account the background.

TABLE 6

	1:20 Dilution (RFU)	1:40 Dilution (RFU)	1:80 Dilution (RFU)
Mumps Virus	13681	9524	5061

RFU was adjusted to take into account the background.

Dilution effects were observed for all three viruses. This is observed by the overall decrease in signal as the dilution is increased. Thus, the induction of caspase 3 activity is affected by the multiplicity of infection used to infect the cells. Because

signal output is affected through dilutions, it is important to have the same multiplicity of infection for direct comparisons between vaccine formulations.

Detection of Differences in Signal Derived from Various Lyophilized Samples

The ability of the caspase 3 assay to detect the effect of different sample and storage conditions was measured to determine the suitability of using the caspase 3 assay to measure the stability of viral preparations stored under different conditions. Measles virus was used to infect cells.

The data shown in Figure 3 illustrates that the effect of different conditions on viral stability can be measured by measuring caspase 3 activity. Thus, the caspase 3 assay can detect differences in viral potency and stability due to various formulation composition and storage conditions.

Correlation Between the Plaque and Caspase 3 Assays

A stability study was performed to examine the correlation between the plaque and caspase 3 assays using measles virus, mumps virus, and rubella virus. The data shown in Figures 4-6 illustrate that as the PFU potency decreased, the corresponding caspase 3 signal also decreased.

Other embodiments are within the following claims. While several embodiments have been shown and described, various modifications may be made without departing from the spirit and scope of the present invention.

WHAT IS CLAIMED

1. A method for assaying potency and stability of a virus comprising the steps of:

a) contacting a plurality of cells susceptible to caspase 3 induction with said virus; and

b) measuring caspase 3 activity as an indication of viral stability.

2. The method of claim 1, wherein said caspase 3 activity is measured using a caspase 3 substrate linked to a fluorimetric or a colorimetric moiety.

3. The method of claim 2, wherein said substrate is the peptide Asp-Glu-Val-Asp.

4. The method of claim 3, wherein said virus is either measles virus, mumps virus, or rubella virus.

5. The method of claim 4, wherein said plurality of cells is either Vero cells or RK-13 cells.

6. The method of claim 3, wherein prior to said step (a) said virus was lyophilized.

7. The method of claim 3, wherein said step (a) and said step (b) are performed at two or more time intervals.

8. The method of claim 3, wherein after said step (a) and prior to said step (b) said cells were frozen and then thawed.

9. A method of identifying a stabilizing formulation comprising the steps of:

a) infecting a first plurality of cells with said virus stored in a first test formulation, and contacting a second plurality of cells with said virus stored in a second test formulation, wherein said first and second plurality of cells are the same strain and are susceptible to caspase 3 induction; and

b) measuring caspase 3 activity produced from said virus stored in said first and said second test formulation to identify said stabilizing formulation.

10. The method of claim 9, wherein said step (a) further comprises contacting a third plurality of cells with said virus stored in a third test formulation, and contacting a fourth plurality of cells with said virus stored in a fourth test formulation, wherein said third and fourth plurality of cells are the same strain as said first and second plurality of cells.

11. The method of claim 10, wherein said caspase 3 activity is measured using a caspase 3 substrate linked to a fluorimetric or a colorimetric moiety.

12. The method of claim 11, wherein said substrate is the peptide Asp-Glu-Val-Asp.

13. The method of claim 12, wherein said virus is either measles virus, mumps virus, or rubella virus.

14. The method of claim 13, wherein said first, said second, said third, and said fourth plurality of cells is either Vero cells or RK-13 cells.

15. The method of claim 12, wherein said virus stored in said first formulation, said second formulation, said third formulation and said fourth formulation was lyophilized.

16. The method of claim 12, wherein said step (a) and said step (b) are performed at two or more time intervals.

17. The method of claim 16, wherein after said step (a) and prior to said step (b) at least one of said first, said second, said third, or said fourth plurality of cells are frozen and thawed.

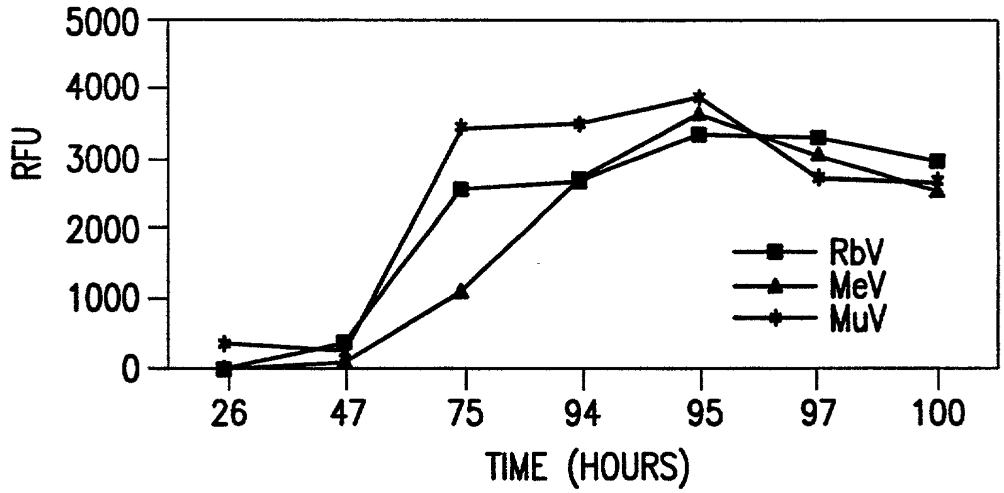


FIG. 1a

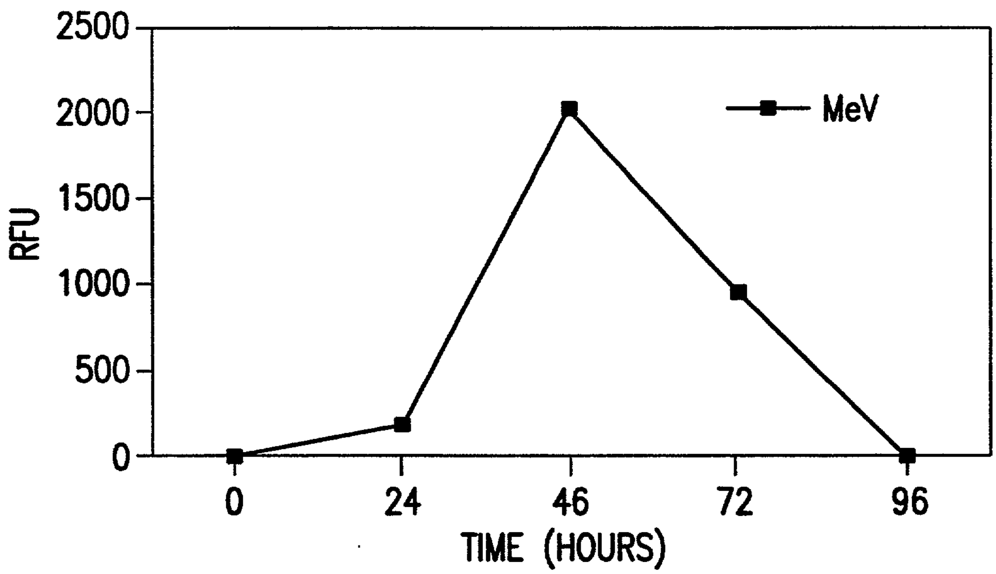


FIG. 1b

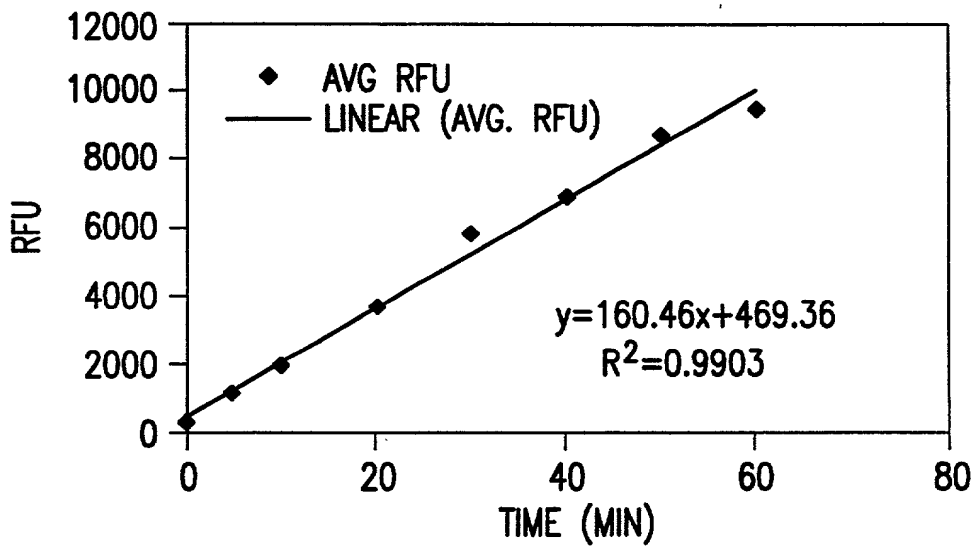
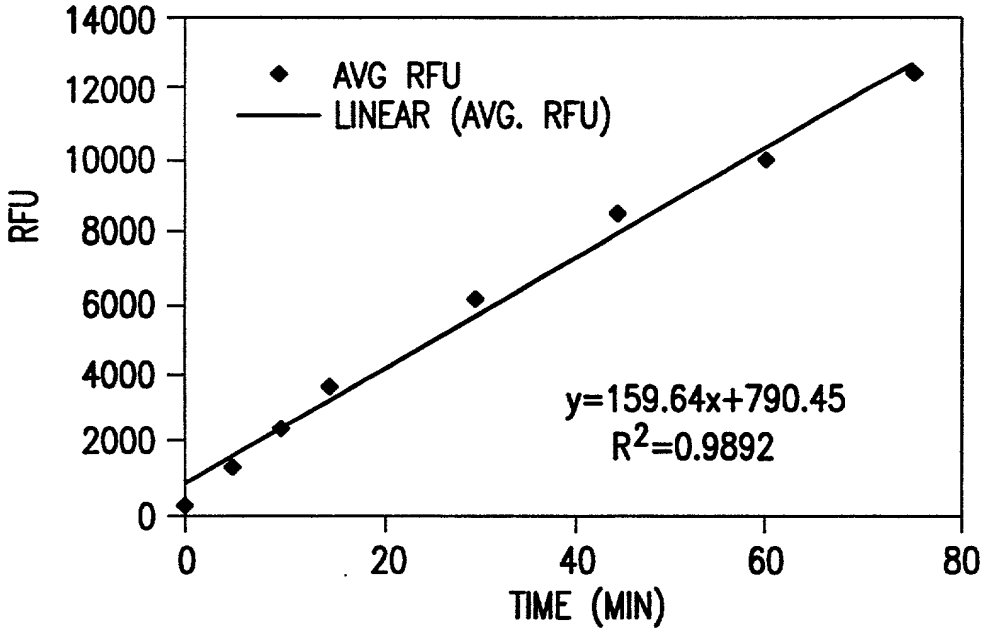

12000
10000
8000
6000
4000
2000
0
RFU
AVG RFU
LINEAR (AVG. RFU)
y=160.46x+469.36
R2=0.9903
0
20
40
60
80
TIME (MIN)

FIG. 2A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12000
10000
8000
6000
4000
2000
0
RFU
AVG RFU
LINEAR (AVG. RFU)
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R2=0.9892
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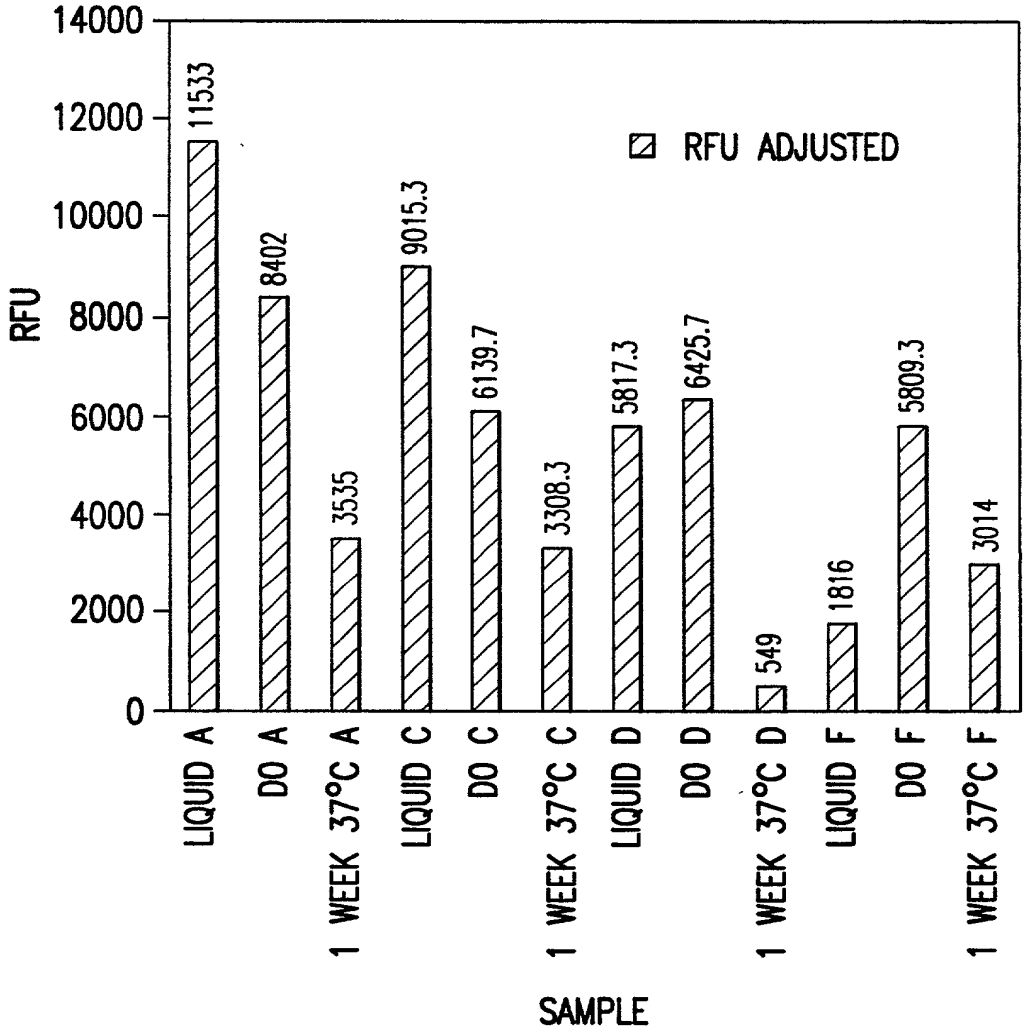
20
40
60
80
TIME (MIN)

FIG. 2B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12000
10000
8000
6000
4000
2000
0
RFU
RFU ADJUSTED
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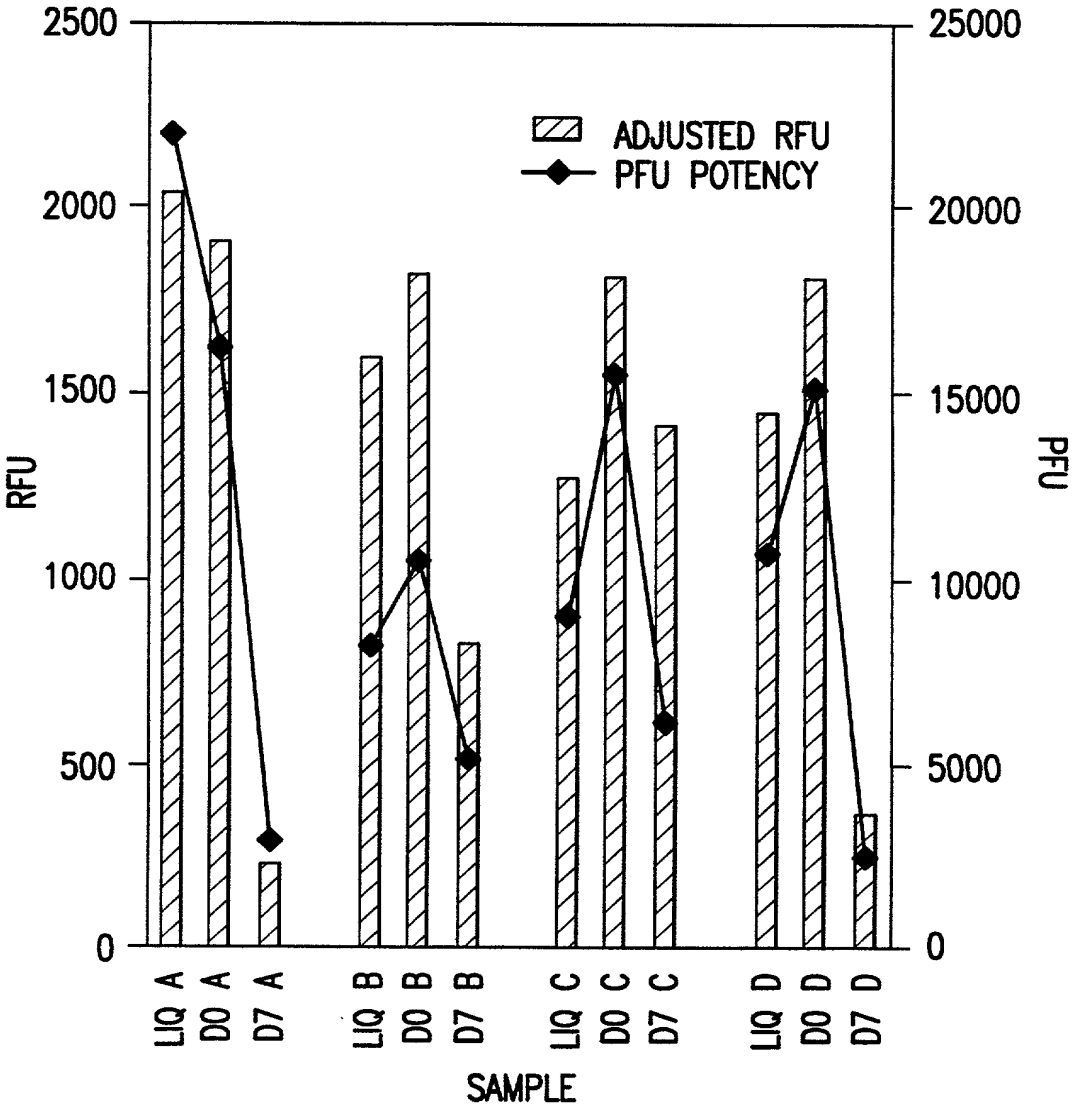
8402
3535
9015.3
6139.7
3308.3
5817.3
6425.7
549
1816
5809.3
3014
LIQUID A
DO A
1 WEEK 37°C A
LIQUID C
DO C
1 WEEK 37°C C
LIQUID D
DO D
1 WEEK 37°C D
LIQUID F
DO F
1 WEEK 37°C F
SAMPLE

FIG.3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2000
1500
1000
500
0
RFU
25000
20000
15000
10000
5000
0
PFU
ADJUSTED RFU
PFU POTENCY
LIQ A
D0 A
D7 A
LIQ B
D0 B
D7 B
LIQ C
D0 C
D7 C
LIQ D
D0 D
D7 D
SAMPLE

FIG.4

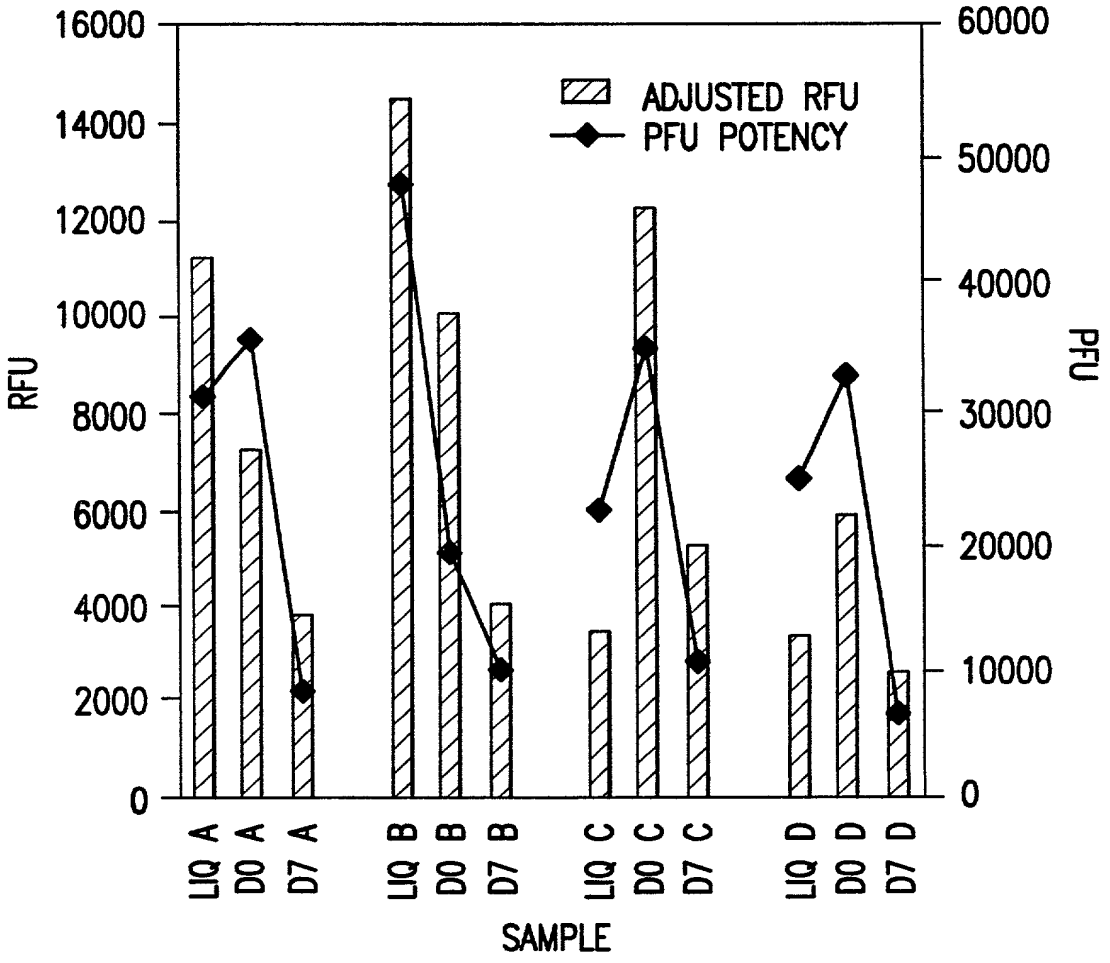
16000
14000
12000
10000
8000
6000
4000
2000
0
RFU
60000
50000
40000
30000
20000
10000
0
PFU
ADJUSTED RFU
PFU POTENCY
LIQ A
D0 A
D7 A
LIQ B
D0 B
D7 B
LIQ C
D0 C
D7 C
LIQ D
D0 D
D7 D
SAMPLE

FIG.5

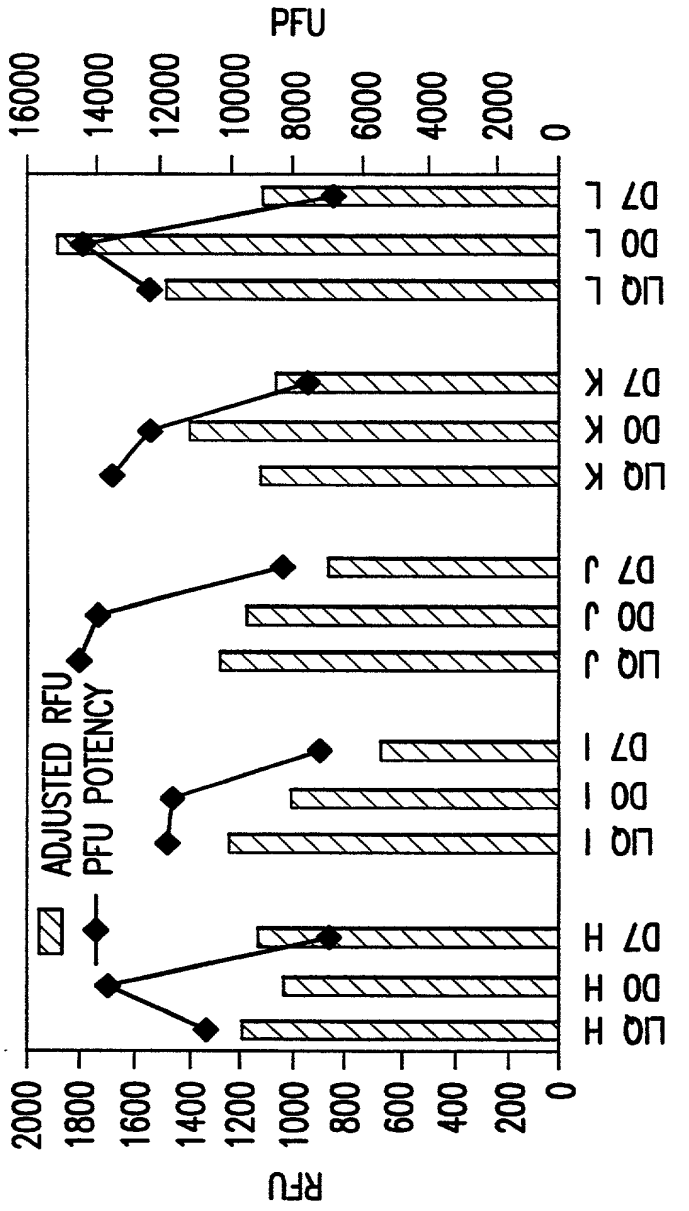
PFU
16000
14000
12000
10000
8000
6000
4000
2000
0
ADJUSTED RFU
PFU POTENCY
LIQ H
DO H
D7 H
LIQ I
DO I
D7 I
LIQ J
DO J
D7 J
LIQ K
DO K
D7 K
LIQ L
DO L
D7 L
SAMPLE
RFU
2000
1800
1600
1400
1200
1000
800
600
400
200
0

FIG.6

Please type a plus sign (+) inside this box → +

Approved for use through 9/30/2000 OMB 651-0032
SUBSTITUTE for PTO/SB/01 (12-97), DECLARATION FOR UTILITY OR DESIGN PATENT APPLICATION

DECLARATION AND POWER OF ATTORNEY FOR UTILITY OR DESIGN PATENT APPLICATION (37 CFR 1.63)

☒ Declaration Submitted with Initial Filing **OR** ☐ Declaration Submitted after Initial Filing (surcharge (37 CFR 1.16 (e)) required)

Attorney Docket Number	20455P
First Named Inventor	Jeffrey T. Blue
COMPLETE IF KNOWN	
Application Number	
Filing Date	
Group Art Unit	
Examiner Name	

As a below named inventor, I hereby declare that:

My residence, post office address, and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

DETECTION OF VIRAL STABILITY

(Title of the Invention)

the specification of which

☐ is attached hereto

OR

☒ was filed on (MM/DD/YYYY) 05/10/2000 as United States Application Number or PCT International Application Number PCT/US00/12638 and was amended on (MM/DD/YYYY) _____ (if applicable).

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment specifically referred to above.

I acknowledge the duty to disclose to the Patent and Trademark Office all information known to me to be material to patentability as defined in 37 CFR 1.56.

I hereby claim foreign priority benefits under 35 U.S.C. 119(a)-(d) or 365(b) of any foreign application(s) for patent or inventor's certificate, or 365(a) of any PCT international application which designated at least one country other than the United States of America, listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate, or of any PCT international application having a filing date before that of the application on which priority is claimed.

Prior Foreign Application Number(s)	Country	Foreign Filing Date (MM/DD/YYYY)	Attorney Docket Number	Priority Claimed? YES	NO
				☐	☐
				☐	☐
				☐	☐
				☐	☐

☐ Additional foreign application numbers are listed on a supplemental priority data sheet PTO/SB/02B attached hereto:

I hereby claim the benefit under 35 U.S.C. 119(e) of any United States provisional application(s) listed below.

Application Number(s)	Filing Date (MM/DD/YYYY)	Attorney Docket Number
60/134,163	05/14/1999	20455PV

Please type a plus sign (+) inside this box → [+]

Approved for use through 9/30/2000 OMB 651-0032

SUBSTITUTE for PTO/SB/01 (12-97), DECLARATION FOR UTILITY OR DESIGN PATENT APPLICATION

DECLARATION AND POWER OF ATTORNEY for Utility or Design Patent Application

I hereby claim the benefit under 35 U.S.C 120 of any United States application(s), or 365(c) of any PCT international application designating the Umted States of America, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT international application in the manner provided by the first paragraph of 35 U.S.C. 112, I acknowledge the duty to disclose information known to me to be material to patentability as defined in 37 CFR 1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application.

U.S. Parent Application or PCT Parent Application Number	Parent Filing Date (MM/DD/YYYY)	Parent Patent Number (if applicable)
PCT/US00/12638	05/10/2000	WO 00/70081

☐ Additional U.S. or PCT international application numbers are listed on a supplemental priority data sheet PTO/SB/02B attached hereto

As a named inventor, I hereby appoint, respectively and individually, as my attorneys or agents with full power of substitution and revocation, the following registered practitioner(s) to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith:

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OR

[X] Registered practitioner(s) name/registration number listed below

Name	Registration Number	Name	Registration Number
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I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under 18 U.S.C. 1001 and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Name of Sole or First Inventor: ☐ A petition has been filed for this unsigned inventor

Given Name (first and middle [if any])	Family Name or Surname
Jeffrey T.	Blue

Inventor's Signature	Jeffrey T. Blue				**Date**	25 Oct 2001	
Residence: City	Hatfield	**State**	PA	**Country**	USA	**Citizenship**	US
Post Office Address	Merck & Co., Inc., P.O. Box 2000						
City	Rahway	**State**	NJ	**ZIP**	07065-0907		

☐ Additional inventors are being named on the ____ supplemental Additional Inventors(s) sheet(s) PTO/SB/02A attached hereto.